

COT102 INSECTICIDAL COTTON

5 The present invention relates to genetic engineering of plants and in particular to an insect resistant transgenic cotton plant. It also relates to methods of detecting material derived from the plant.

10 Plant pests are a major factor in the loss of the world's important agricultural crops. About \$8 billion is lost every year in the U.S. due to infestations of plants by non-mammalian pests including insects. In addition to losses in field crops, insect pests are also a burden to vegetable and fruit growers, to producers of ornamental flowers, and to home gardeners.

15 Insect pests are mainly controlled by intensive applications of chemical pesticides, which are active through inhibition of insect growth, prevention of insect feeding or reproduction, or cause death. Good control of insect pests can thus be reached, but these chemicals can sometimes also affect other, beneficial insects. Another problem resulting from the wide use of chemical pesticides is the appearance of resistant insect varieties. This has been partially alleviated by various resistance management practices, but there is an increasing need for alternative pest control agents. Biological pest control agents, such as *Bacillus*
20 *thuringiensis* strains expressing pesticidal toxins like δ -endotoxins, have also been applied to crop plants with satisfactory results, offering an alternative or complement to chemical pesticides. The genes coding for some of these δ -endotoxins have been isolated and their expression in heterologous hosts has been shown to provide another tool for the control of economically important insect pests. In particular, the expression of insecticidal toxins
25 such as *Bacillus thuringiensis* δ -endotoxins in transgenic plants, has provided efficient protection against selected insect pests, and transgenic plants expressing such toxins have been commercialised, allowing farmers to reduce applications of chemical insect control agents.

30 Recently, a new family of insecticidal proteins produced by *Bacillus* sp. during the vegetative stages of growth (vegetative insecticidal proteins (VIPs)) has been identified. U.S. Patents 5,877,012, 6,107,279, and 6,137,033 describe *vip3A* toxin genes isolated from *Bacillus* species. The VIP3A toxins possess insecticidal activity against a wide spectrum

of lepidopteran insects including but not limited to fall armyworm, *Spodoptera frugiperda*, black cutworm, *Agrotis ipsilon*, sugarcane borer, *Diatraea saccharalis*, and lesser cornstalk borer, *Elasmopalpus lignosellus*, and when expressed in transgenic plants, for example cotton, confer protection on the plant from insect feeding damage.

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The cotton family, genus *Gossypium*, a member of the Malvaceae, consists of 39 species, of which *Gossypium hirsutum* is the most commonly cultivated species. Three other species are also cultivated: *G. arboreum*, *G. barbadense*, and *G. herbaceum*. These cultivated species are grown primarily for the seed hairs that are made into textiles. Cotton is suitable as a textile fibre because the mature dry hairs twist in such a way that fine strong threads can be spun from them. Other products, such as cottonseed oil, cake, and cotton linters are by-products of fibre production.

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Damage to cotton crops by insect pests throughout the world results in a significant yield loss each year. Effective control of these pests to minimise yield loss is of great economic importance. Examples of insect pests of cotton include Beet armyworm (*Spodoptera exigua*), Boll weevil (*Anthonomus grandis grandis*), Cabbage looper (*Trichoplusia ni*), Clouded plant bug (*Neurocolpus nubilus*), Cotton aphid (*Aphis gossypii*), Cotton bollworm (*Heliocoverpa zea*), Cutworms (*Feltia subterranea*, *Peridroma saucia*, *Agrotis ipsilon*), European corn borer (*Ostrinia nubilalis*), Fall armyworm (*Spodoptera frugiperda*), Seedling thrips (*Frankliniella* spp.), Soybean looper (*Pseudoplusia includens*), Stink bugs (*Nezara viridula*, *Acrosternum hilare*, *Euschistus servus*), Tarnished plant bug (*Lygus lineolaris*), Tobacco budworm (*Heliothis virescens*) and Whiteflies (*Trialeurodes abutilonea*, *Bemisia tabaci*).

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Transformation and regeneration of cotton plants is now a well-established procedure, typically based on *Agrobacterium tumefaciens* mediated transfer of foreign DNA into cotton plant parts and regeneration of said plant parts in tissue culture into fully fertile, transgenic cotton plants.

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There exists a requirement to generate a cotton plant that is insect resistant so that yield loss through damage to cotton crops by insect pests is reduced. An insect resistant cotton

plant could reduce the need to apply chemical pesticides, which may be detrimental to other, beneficial insects and the environment.

Therefore, the present invention relates to an insect resistant transgenic cotton event, designated COT102. It also relates to methods of detecting plant material derived therefrom. "COT102 event" in the context of this application refers to the original insecticidal transgenic cotton plant described herein. "Insecticidal" as used herein refers to any inhibitory effect on an insect, including but not limited to reduced feeding, retarded growth, reduced fecundity, paralysis or death. "Fecundity" comprises all aspects related to reproduction such as reproductive ability, reproductive frequency and number of offspring. Also embraced by this invention is any plant material derived from the COT102 event, including seeds.

The COT102 event exhibits a novel genotype comprising two expression cassettes. The first cassette comprises a suitable promoter for expression in plants operably linked to a gene that encodes a VIP3A insecticidal toxin, useful in controlling a wide spectrum of lepidopteran insect pests, and a suitable polyadenylation signal. Suitable promoters may be isolated from, *inter alia*, plants. Numerous plant promoters have been isolated and characterised including constitutive, switchable and/or tissue specific promoters. Suitable promoters may be selected from the following, non-limiting group: CaMV35S, FMV35S, Ubiquitin, Act2, NOS, OCS, Cestrum yellow leaf curl virus promoter, Patatin, E9, alcA/alcR switch, GST switch, RMS switch, oleosin, Gelvin, ribulose biphosphate carboxylase-oxygenase small sub-unit, actin 7, MR7 promoter (maize), Gos 9 (rice), GOS2 promoters, MasOcs (or super promoter), RolD promoter (*Agrobacterium rhizogenes*), SuperMAS promoter, and Suc2 promoter (Arabidopsis). In one embodiment of the present invention, the promoter is the Actin promoter, Act2, from Arabidopsis. Additional elements such as enhancer sequences may also be incorporated into the expression cassette in order to boost levels of gene expression, for example transcriptional or translational enhancers, such as tobacco etch virus (TEV) translation activator, CaMV35S enhancer, and FMV35S enhancer. Alternatively it may be desirable to include a targeting sequence, for example, to direct transportation of the VIP3A toxin to a particular cellular compartment. For example if it is desired to provide the protein outside of the cell then an extracellular targeting sequence may be ligated to the polynucleotide encoding the VIP protein. Other

examples of targeting include targeting to a specific intracellular organelle or compartment, for example to the endoplasmic reticulum using a 'KDEL' retention sequence. Numerous polyadenylation signals have been isolated and characterised. Examples of suitable polyadenylation signals functional in plants include that from the
5 nopaline synthase gene (nos) of *Agrobacterium tumefaciens*, from the proteinase inhibitor II gene and from the alpha-tubulin gene (EP-A 652,286). In one embodiment of the present invention, the polyadenylation signal is that from the nos gene of *Agrobacterium tumefaciens*.

10 According to the invention, the polynucleotide encoding the VIP3A protein may also be codon-optimised or otherwise altered to enhance for example, transcription once it is incorporated into plant material. Such codon optimisation may also be used to alter the predicted secondary structure of the RNA transcript produced in any transformed cell, or to destroy cryptic RNA instability elements present in the unaltered transcript, thereby
15 increasing the stability and/or availability of the transcript in the transformed cell (Abler and Green (1996) Plant Molecular Biology (32) pp.63-78).

The second cassette comprises a gene which, when expressed, can be used as a selectable marker. Numerous selectable markers have been characterised, including some that confer
20 tolerance to antibiotics and others that confer tolerance to herbicides. Examples of suitable selectable marker genes include those that confer tolerance to- hygromycin, kanamycin or gentamycin. Further suitable selectable markers include genes that confer resistance to herbicides such as glyphosate-based herbicides or resistance to toxins such as eutypine. Other forms of selection are also available such as hormone based selection systems such
25 as the Multi Auto Transformation (MAT) system of Hiroyasu Ebinuma *et al.* (1997) PNAS Vol. 94 pp.2117-2121; visual selection systems which use the known green fluorescence protein, β glucuronidase and any other selection system such as mannose isomerase (PositechTM), xylose isomerase and 2-deoxyglucose (2-DOG). In one embodiment of the present invention, the selectable marker gene is one that confers
30 tolerance to hygromycin. Further expression cassettes are optionally comprised in the COT102 event. For example these may provide other desirable benefits such as herbicide resistance.

The first and second expression cassettes may be introduced into the plant on the same or different plasmids. If the first and second expression cassettes are present on the same plasmid and introduced into the plant via an *Agrobacterium*-mediated transformation method, they may be present within the same or different T-DNA regions. In one
5 embodiment of the present invention, the first and second expression cassettes are present on the same T-DNA region.

According to the first aspect of the invention, there is provided a polynucleotide comprising at least 17 contiguous nucleotides from the 26-nucleotide sequence of SEQ ID
10 NO: 1. In one embodiment said polynucleotide comprises at least 18 contiguous nucleotides from SEQ ID NO: 1. In a further embodiment said polynucleotide comprises at least 20 contiguous nucleotides from SEQ ID NO: 1. In a still further embodiment said polynucleotide comprises at least 22 contiguous nucleotides from SEQ ID NO: 1. In yet a further embodiment said polynucleotide comprises at least 24 contiguous nucleotides from
15 SEQ ID NO: 1. In a still further embodiment there is provided a polynucleotide comprising the sequence of SEQ ID NO: 1.

In a further aspect of the invention, there is provided a polynucleotide comprising at least 17 contiguous nucleotides from the 26-nucleotide sequence of SEQ ID NO: 2. In one
20 embodiment said polynucleotide comprises at least 18 contiguous nucleotides from SEQ ID NO: 2. In a further embodiment said polynucleotide comprises at least 20 contiguous nucleotides from SEQ ID NO: 2. In a still further embodiment said polynucleotide comprises at least 22 contiguous nucleotides from SEQ ID NO: 2. In yet a further embodiment said polynucleotide comprises at least 24 contiguous nucleotides from SEQ
25 ID NO: 2. In a still further embodiment there is provided a polynucleotide comprising the sequence of SEQ ID NO: 2.

In a further aspect of the present invention there is provided a polynucleotide as described above comprising the sequence of SEQ ID NO: 7. In a still further aspect of the present
30 invention, there is provided a polynucleotide as described above comprising the sequence of SEQ ID NO: 21.

In another aspect of the present invention there is provided a plant comprising a polynucleotide which comprises at least 17 contiguous nucleotides of SEQ ID NO: 1 and / or SEQ ID NO: 2. In one embodiment said plant comprises at least 18 contiguous nucleotides of SEQ ID NO: 1 and / or SEQ ID NO: 2. In a further embodiment said plant
5 comprises at least 20 contiguous nucleotides of SEQ ID NO: 1 and / or SEQ ID NO: 2. In a further embodiment said plant comprises at least 22 contiguous nucleotides of SEQ ID NO: 1 and / or SEQ ID NO: 2. In a still further embodiment said plant comprises at least 24 contiguous nucleotides of SEQ ID NO: 1 and / or SEQ ID NO: 2. In yet a further embodiment said plant comprises the sequence of SEQ ID NO: 1 and / or SEQ ID NO: 2.
10 In a further embodiment still, said plant additionally comprises the sequence of SEQ ID NO: 7. In another embodiment, said plant comprises the sequence of SEQ ID NO: 21. In one embodiment of the present invention, said plant is a cotton plant. In a further embodiment, said plant is an insecticidal cotton plant which is the COT102 event, or a plant derived therefrom.

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The skilled man is familiar with plant transformation methods. In particular, two principal techniques have been characterised across a wide range of plant species: transformation by *Agrobacterium* and transformation by direct DNA transfer.

20 *Agrobacterium*-mediated transformation is a commonly used method for transformation of dicotyledonous plants. The foreign DNA to be introduced into the plant is cloned into a binary vector in between left and right border consensus sequences. This is the T-DNA region. The binary vector is transferred into an *Agrobacterium* cell, which is subsequently used to infect plant tissue. The T-DNA region of the vector comprising the foreign DNA
25 is inserted into the plant genome. The marker gene cassette and trait gene cassette may be present on the same T-DNA region, different T-DNA regions in the same vector, or even different T-DNA regions in different vectors. In one embodiment of the present invention, the cassettes are present on the same T-DNA region.

30 Alternatively, direct DNA transfer can be used to introduce the DNA directly into a plant cell. One suitable method of direct transfer may be bombardment of plant cells with a vector comprising the DNA for insertion using a particle gun (particle-mediated biolistic transformation); another established method, 'whiskers', involves coating the DNA onto

silicon carbide fibres onto which cells are impaled. Other methods for transforming plant cells include protoplast transformation (optionally in the presence of polyethylene glycols); sonication of plant tissues, cells or protoplasts in a medium comprising the polynucleotide or vector; micro-insertion of the polynucleotide or vector into plant material (optionally
5 employing the known silicon carbide "whiskers" technique), electroporation and the like.

Following transformation, transgenic plants must be regenerated from the transformed plant tissue, and progeny possessing the foreign DNA selected using an appropriate marker such as resistance to hygromycin. The skilled man is familiar with the composition of
10 suitable regeneration media.

A plant of this aspect of the invention, as described herein, has an insecticidal effect on insects from one or more species from the group comprising *Heliothis* sp., *Helicoverpa* sp. and *Spodoptera* sp. which may infest it. "Infest" as used herein refers to attack, feeding or
15 damage in any way by one or more insects. Thus, for example, the plant of the present invention will provide a self-defence mechanism against infestation by pest insects such as *Helicoverpa zea* (cotton boll worm). As a result, a reduced number of insecticide sprays are required during the cultivation of said plant compared to a non-transgenic cotton plant of the same variety and yield loss through insect pests is kept at a minimal level.

20 The present invention is not limited to the COT102 event itself, but is further extended to include any plant material derived therefrom, including seeds in so far as they contain at least one of the present inventive polynucleotides. The present invention includes, but is not limited to plants that are derived from a breeding cross with the COT102 event or a
25 derivative therefrom by conventional breeding or other methods. The invention also includes plant material derived from the COT102 event that may comprise additional, modified or fewer polynucleotide sequences compared to the COT102 event or exhibit other phenotypic characteristics. For example it may be desirable to transform the plant material derived from the COT102 event to generate a new event that possesses an
30 additional trait, such as a second insect resistance gene. This process is known as gene stacking. The second insect resistance gene may encode, for example insecticidal lectins, insecticidal protease inhibitors and insecticidal proteins derived from species of the *Bacillus thuringiensis*, *Xenorhabdus nematophilus*, or *Photorhabdus luminescens*.

Preferably, the second insect resistance gene encodes a Cry gene from the bacterium *Bacillus thuringiensis*, which Cry gene produces a toxin with a different mode of action or binding site in the insect gut to VIP for the control of different insect species.

The present invention further provides plant material derived from the COT102 event
5 which possesses an additional trait such as herbicide resistance, nematode resistance or fungal resistance. In one embodiment, said additional trait is herbicide resistance. In a further embodiment, said herbicide resistance trait provides resistance to a herbicide which comprises glyphosate acid or an agriculturally acceptable salt thereof. In a further
10 embodiment still, said herbicide resistance trait is provided by a gene encoding EPSP synthase or a mutant thereof.

The present invention further provides a method of controlling insects comprising providing plant material derived from the COT102 event at a locus where said insects feed. The invention yet further provides a method of controlling insects comprising providing
15 plant material derived from the COT102 event at a locus where said insects feed, and applying other agrochemicals to said plant material such as herbicides, fungicides and other insecticidal compounds including other insecticidal proteins. Examples of possible insecticidal compounds include insecticidal lectins, insecticidal protease inhibitors and insecticidal proteins derived from species of the *Bacillus thuringiensis*, *Xenorhabdus*
20 *nematophilus*, or *Photorhabdus luminescens*. Examples of possible chemicals include pyrethroids, carbamates, imidacloprid, organochlorines, and macromolecules such as spinosad, abamectin or emamectin.

According to yet a further aspect of the present invention, there is provided a method of
25 detecting plant material derived from the COT102 transgenic event comprising obtaining a sample for analysis; extracting DNA from the sample; providing a pair of primers designed to bind to a polynucleotide comprising at least 17 contiguous nucleotides of SEQ ID NO: 1 and / or SEQ ID NO: 2; amplifying the region which lies between the sites at which the primers bind; and detecting the presence of the amplification product. Suitable pairs of
30 primers for use in this method of detection can be designed using parameters well known to those skilled in the art of molecular biology now that SEQ ID NOs 1 and 2 are made available. For example, one or both primers of the pair may be designed to be vector-specific, trait gene specific, promoter specific, specific to the sequence of the junction

between the inserted DNA and the genomic DNA, and/or marker specific. In one embodiment, the sequence of said primers is depicted as SEQ ID NO: 3 and SEQ ID NO: 4.

- 5 In an embodiment of the present invention, the region amplified by said method (the 'amplicon') is between 300 and 1000 base pairs in length. In a further embodiment the amplicon is between 500 and 900 base pairs in length. In a still further embodiment the amplicon is 800 base pairs in length. In a further embodiment the amplicon is produced using the above method in conjunction with the primers of the sequence of SEQ ID NO: 3
10 and SEQ ID NO: 4, and is 800 base pairs in length.

- Alternative primers which may be used in combination to detect the COT102 event include SEQ ID NOs 18 and 19 which are specific for the COT102 event and produce a 962bp amplicon, SEQ ID NOs 22 and 23 which are specific for the VIP gene and produce a
15 556bp amplicon, or SEQ ID NOs 24 and 25 which are specific for the gene conferring resistance to the antibiotic hygromycin and produce a 367bp amplicon.

- There are many amplification methods that may be used in accordance with this aspect of the invention. The underlying principle, a known technique to those skilled in the art, is
20 the polymerase chain reaction (PCR). The amplification product from a PCR reaction may be visualised by staining with ethidium bromide and excitation with UV light, typically after size separation using agarose gel electrophoresis.

- An embodiment of the present invention employs variations of the PCR principle such as
25 TaqMan™. This involves labelling at least one of the primers involved in the amplification process with a fluorescent dye. When unbound, the primer adopts a conformation such that no fluorescence can be detected. However, when the primer is bound to a piece of DNA, the conformation changes and fluorescence can be detected. In this way, the amplification process can be monitored in real-time, the intensity of
30 fluorescence corresponding directly to the level of amplification. Further embodiments of the present invention include, but are not limited to, RACE PCR.

A further embodiment of the present invention involves the use of multiplex PCR for distinguishing between homozygous COT102 plant material and heterozygous COT102 plant material. This is known to those skilled in the art as zygoty testing, and involves the use of three PCR primers which bind to specific parts of the cotton genome and / or
5 inserted DNA. Suitable primers for use in such a zygoty test are depicted as SEQ ID NOs 18 to 20.

In another aspect of the invention there is provided a method of detecting plant material derived from the COT102 event comprising obtaining a sample for analysis; providing a
10 probe designed to bind to the complement of a polynucleotide which comprises at least 17 contiguous nucleotides of SEQ ID NO: 1 and / or SEQ ID NO: 2 when said polynucleotide is single stranded; hybridising said probe with the sample; and detecting whether the probe has hybridised. In one embodiment, said probe comprises the sequence of SEQ ID NO: 1 and / or SEQ ID NO: 2. In an embodiment of the present invention there is provided a
15 method of detecting plant material derived from the COT102 event using a probe selected from the group comprising SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7. In one embodiment, said probe comprises SEQ ID NO: 5. In a further embodiment, said probe consists of SEQ ID NO: 5. The probe may be, for example, a PCR product or restriction digestion fragment. In a further embodiment, the probe as described herein may be tagged
20 with a fluorescent, radioactive, enzymatic or other suitable label to enable hybridisation to be detected. The skilled man will know how to design suitable probes, now that he has the benefit of the present disclosure.

In a further embodiment of the present invention, there is provided a method of hybridising
25 a probe to the sample under stringent conditions and detecting whether the probe has hybridised. Stringent hybridisation conditions are well known to the skilled man and comprise, for example: hybridisation at a temperature of about 65°C in a solution containing 6 x SSC, 0.01% SDS and 0.25% skimmed milk powder, followed by rinsing at the same temperature in a solution containing 0.2 x SSC and 0.1% SDS.

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Suitable techniques for detecting plant material derived from the COT102 event based on the hybridisation principle include, but are not limited to Southern Blots, Northern Blots and *in-situ* hybridisation. The skilled man is familiar with techniques such as these.

Typically, they involve incubating a probe with a sample, washing to remove unbound probe, and detecting whether the probe has hybridised. Said detection method is dependent on the type of tag attached to the probe – for example, a radioactively labelled probe can be detected by exposure to and development of x-ray film. Alternatively, an enzymatically labelled probe may be detected by conversion of a substrate to effect a colour change.

In a further aspect of the invention there is provided a method of detecting plant material derived from the COT102 event comprising obtaining a sample for analysis; providing an antibody designed to bind to a VIP protein contained within a plant comprising at least 17 contiguous nucleotides from SEQ ID NO: 1 and / or SEQ ID NO: 2; incubating said antibody with the sample; and detecting whether the antibody has bound. In one embodiment of the present invention said VIP protein comprises the sequence of SEQ ID NO: 8.

Suitable methods of detecting plant material derived from the COT102 event based on said antibody binding include, but are not limited to Western Blots, Enzyme-Linked ImmunoSorbent Assays (ELISA) and SELDI mass spectrometry. The skilled man is familiar with these immunological techniques. Typical steps include incubating a sample with an antibody that binds to the VIP protein, washing to remove unbound antibody, and detecting whether the antibody has bound. Many such detection methods are based on enzymatic reactions - for example the antibody may be tagged with an enzyme such as horse radish peroxidase, and on application of a suitable substrate, a colour change detected. Suitable antibodies may be monoclonal or polyclonal.

In another aspect of the invention there is provided a method of detecting plant material derived from the COT102 event comprising obtaining a sample for analysis; making a protein extract of the sample; providing a test strip designed to detect the presence of a VIP protein present within the sample; incubating the test strip with the sample; and detecting whether VIP protein is present. In one embodiment of the present invention said VIP protein comprises the sequence of SEQ ID NO: 8.

An alternative antibody-based detection method for COT102 uses of dipsticks or test strips. Typical steps include incubating a test strip with a sample and observing the presence or absence of coloured bands on the test strip. The coloured bands are indicative of the presence of a protein in the sample. Such dipstick or test strip tests are protein specific, and may be used for rapid testing of samples in the field.

In a further aspect of the present invention there is provided a method of detecting plant material derived from the COT102 event comprising obtaining a sample for analysis; subjecting one or more insects of the species *Spodoptera frugiperda* (susceptible to VIP3A) to the sample; subjecting one or more insects of species *Ostrinia nubilalis* (not susceptible to VIP3A) to the sample as a control; detecting whether the sample has an insecticidal effect on insects from each species; and comparing the results with an authentic COT102 bioassay profile. The results are compared against an authentic COT102 bioassay profile that is produced using insects of the same condition which have been subjected to the same dose and type of COT102 plant material and where the insecticidal effect is detected the same length of time after subjecting the insects to the COT102 sample. *Spodoptera frugiperda* is a positive control for COT102 as it is susceptible to a suitable dose of VIP3A, while *Ostrinia nubilalis* is a negative control for COT102 as it is not susceptible to a suitable dose of VIP3A.

In one embodiment of the invention, the method of detecting plant material derived from the COT102 event includes but is not limited to leaf-feeding bioassays in which a leaf or other suitable plant part from the COT102 event or any plant material derived from the COT102 event, is infested with one or more pest insects. Detection may be through assessment of damage to the leaf or plant part after set time periods, assessment of mortality or another insecticidal effect on the insects. Alternative plant parts which may be used for such bioassays include bolls and squares. Such bioassays may be carried out in the field, or glasshouse, and may be subject to natural or artificial insect infestation.

In another aspect of the invention, there is provided a kit of parts comprising a means for detecting the presence in a sample of plant material derived from the COT102 event. Preferably, said kit of parts comprises a means for detecting the presence in a sample of a polynucleotide comprising at least 17 contiguous nucleotides from the sequence of SEQ ID

NO: 1 and / or SEQ ID NO: 2, or a protein encoded by a polynucleotide as described above, or a VIP protein. In an embodiment of the present invention, said kit of parts may comprise DNA amplification-detection technology such as PCR or TaqMan™. In a further embodiment of the present invention, said kit of parts may comprise probe hybridisation-
5 detection technology such as Southern Blots, Northern Blots or *in-situ* Hybridisation. In another embodiment of the present invention, said kit of parts may comprise antibody binding-detection technology such as Western Blots, ELISA's, SELDI mass spectrometry or test strips. In a further embodiment of the present invention, said kit of parts may
10 comprise insect bioassay-detection technology such as leaf feeding bioassays or mortality bioassays. In a further embodiment of the present invention, said kit of parts may comprise any combination of the afore-mentioned detection technologies. In a still further embodiment, said kit of parts may comprise in the form of instructions one or more of the methods described above.

15

EXAMPLES

The invention will be further apparent from the following non-limiting examples in conjunction with the associated sequence listings as described below:

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SEQ ID NO 1: Polynucleotide sequence which extends across the junction where the 5' end of the COT102 insert is inserted into the cotton genome in event COT102.

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SEQ ID NO 2: Polynucleotide sequence which extends across the junction where the 3' end of the COT102 insert is inserted into the cotton genome in event COT102.

SEQ ID NOs 3 - 4: Polynucleotide sequences suitable for use as primers in the detection of the COT102 event.

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SEQ ID NOs 5 - 7: Polynucleotide sequences suitable for use as probes in the detection of the COT102 event.

SEQ ID NO 8: Amino acid sequence of the VIP3A toxin protein.

SEQ ID NOs 9 - 17: Polynucleotide sequences suitable for use as TaqMan primers in the detection of the COT102 event.

SEQ ID NOs 18 - 20: Polynucleotide sequences suitable for use as primers in the detection of the COT102 event via zygosity testing.

SEQ ID NO 21: Polynucleotide sequence characterising COT102 event.

5 SEQ ID Nos 22 – 25: Polynucleotide sequences suitable for use as primers in the detection of the COT102 event.

Example 1: Cloning and Transformation

1.1 Vector cloning

Standard gene cloning techniques of restriction digestion and ligation of fragments from
10 in-house vectors were used to construct the transformation vector, pNOV3001. The vector included a selectable marker cassette comprising a Ubiquitin (UBQ3) promoter, the UBQ3 intron, a gene sequence which encodes a protein conferring resistance to hygromycin, and a nos polyadenylation sequence. The vector also included the expression cassette of the target gene, which cassette comprised an Actin (Act2) promoter, the Act2 intron, a
15 sequence encoding the VIP3A gene that had been codon optimised for expression in maize, and a nos polyadenylation sequence. The selectable marker cassette and VIP3A containing cassette were cloned within the T-DNA region of vector pNOV3001, between the left and right border sequences. The vector also comprised a gene conferring resistance to an antibiotic, spectinomycin, for prokaryotic selection.

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The vector was transformed into *Agrobacterium tumefaciens* strain EHA101 using standard *Agrobacterium* transformation techniques, and transformed cells selected through their resistance to spectinomycin.

25 1.2 Plant transformation

The COT102 event was produced by *Agrobacterium*-mediated transformation of *Gossypium hirsutum* L. cv Coker 312.

Coker 312 seeds were surface sterilized for 30 seconds in 70% ethanol using enough
30 ethanol to cover the amount of seed to be sterilized. The seeds were washed with ethanol, rinsed in sterile water and soaked in a 12% Clorox + Tween 20 solution for 20 minutes. This washing procedure was performed 3 times. Seeds were then placed onto germination medium (Stewart and Hsu, 1977) and allowed to germinate at 30°C for 7 – 10 days.

2 ml cultures of *Agrobacterium* containing the pNOV3001 construct were grown overnight in appropriate antibiotics and then diluted with MSNH medium (19:1) in a sterile petri dish. Hypocotyls were cut into 6 – 8 mm lengths and placed in the diluted *Agrobacterium* solution for at least 30 seconds. Hypocotyl explants were removed from the *Agrobacterium* solution and blotted on sterile filter paper to remove excess bacteria. Hypocotyls were placed onto T2 medium (MS salts, B5 vitamins, 0.1 mg/L 2, 4-D, 0.5 mg/L kinetin, 30 g/L glucose, 2 g/L Phytigel – pH 5.8) and co-cultured with the *Agrobacterium* for 72 hours in the dark.

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Hypocotyl explants were again blotted on sterile filter paper and transferred to plates containing MS2NK medium (MS salts, B5 vitamins, 2 mg/L NAA, 0.1 mg/L kinetin, 30 g/L glucose, 2 g/L Phytogel, 500 mg/L cefotaxime, 10 mg/L hygromycin – pH 5.8). The plates were wrapped with parafilm and incubated in the light at 30°C for several months until callus was formed.

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Callus was broken up as small as possible and placed in a 50 ml Erlenmeyer flask containing 10 ml of liquid MSNH medium (MS salts, B5 vitamins, 30 g/L glucose – pH 5.8). The suspended callus was shaken at 110 rpm in the light at 30°C until small white slightly round cell clusters were visible. Cells were washed and plated onto solid MSNH medium (MS salts, B5 vitamins, 30 g/L glucose, 2 g/L Phytogel – pH 5.8). Plates were checked monthly for somatic embryo development.

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Mature somatic embryos were picked from the plates and placed onto plates containing SA medium (Stewart and Hsu salts, 20 g/L sucrose, 20 g/L agar – pH 5.8). The plates of embryos were placed in the dark for approximately 14 days. Roots were trimmed from the maturing embryos and the embryos were transferred to SGA medium (Stewart and Hsu salts, 5 g/L sucrose, 1.5 g/L Phytogel, 5 g/L agar – pH 6.8).

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After the first true leaf emerged, young plants were moved to pint sized canning jars containing SGA medium. When the plants reached 7 – 10 cm in height, the top was cut off and transferred to another jar. On developing a good root system, the thus rooted cuttings were transplanted into pots and grown in the greenhouse.

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1.3 Identification and selection of transgenics

Putative transgenic plants were screened by PCR for the presence of the VIP3A gene. Positive events were identified and screened using insect bioassays for insecticidal activity against Fall Armyworm (*Spodoptera frugiperda*) (see Example 7). Insecticidal lines were characterized for copy number by TaqMan™ analysis (see Example 2). T1 seed from 3 single copy & 2 double copy events were observed in a field trial for insect resistance and agronomic quality. Two events, COT101 and COT102, were chosen based on having a single copy of the transgene, good protein expression as identified by ELISA (see Example 4), good insecticidal activity against Cotton Boll Worm (*Helicoverpa zea*) and field performance. At the end of the second year of field trials, results between the two events were compared and COT102 was progressed.

1.4 Verification of sequence of COT102

Genomic DNA was isolated from the COT102 event. This was used in the sequencing of the junctions of the DNA insertion site with the cotton genomic DNA in the COT102 event, using standard DNA sequencing techniques.

Example 2: COT102 Detection via TaqMan™

2.1 DNA extraction

DNA was extracted from leaf tissue using the Wizard™ Magnetic 96 DNA Plant System (Promega, #FF3760), according to the manufacturers instructions, with an additional step at the beginning of the protocol: following grinding of the leaf material, 0.9ml Cotton Extraction Buffer (0.2M Tris pH 8.0, 50mM EDTA, 0.25M NaCl, 0.1% v/v 2-mercaptoethanol, 2.5% w/v polyvinyl-pyrrolidone) was added to each well, the plant tissue resuspended and the plate centrifuged at 4,000 rpm (2755g) for 10 minutes. After aspirating and discarding the supernatant, 300ul Lysis Buffer A (Promega) was added and the manufacturers protocol was followed from this point. This procedure resulted in approximately 85ul of purified genomic DNA at a concentration of approximately 10ng/ul.

2.2 TaqMan PCR reactions

TaqMan™ PCR reactions were setup using a standard reaction mix comprising:

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- 625ul 2x Jumpstart Master Mix for Q-PCR (Sigma, #P2893), supplemented with
15mM MgCl₂ and 200nM Strata-ROX
- 25ul 50x FAM primer / probe mix
- 25ul 50x TET primer / probe mix
- 5 200ul Water.

50x primer / probe mixes comprise 45ul of each primer at a concentration of 1mM, 50ul of the probe at a concentration of 100uM and 860ul 1x TE, and were stored in an amber tube at 4°C. Examples of suitable primer / probe sequence combinations which were used are:

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<u>Primer Name</u>	<u>Primer Sequence 5'-3'</u>	<u>SEQ ID</u>
GhCHI2b-F Forward	GGTCCCTGGATACGGTGTCA	SEQ ID NO: 9
GhCHI2b-R Reverse	TTGAGGGTTGGATCCTTTGC	SEQ ID NO: 10
GhCHI2b-TET Probe	CCAACATCATCAATGGTGGCATCGAAT (5' label = TET, 3' label = TAMRA)	SEQ ID NO: 11
Hygromycin-F Forward	CAGGCAGGTCTTGCAACGT	SEQ ID NO: 12
Hygromycin-R Reverse	CGAGAGCCTGACCTATTGCAT	SEQ ID NO: 13
Hygromycin-FAM Probe	ACACCCTGTGCACGGCGGG (5' label = FAM, 3' label = TAMRA)	SEQ ID NO: 14
Vip3-F Forward	ATGAAGACCCTGCGCTACGA	SEQ ID NO: 15
Vip3-R Reverse	ACGCCCAGTGGCATGTAGA	SEQ ID NO: 16
Vip3-FAM Probe	AGCGAGGCCGAGTACCGCACC (5' label = FAM, 3' label = TAMRA)	SEQ ID NO: 17

7ul of master mix was dispensed into each well of a 384-well TaqMan™ assay plate. 3ul DNA template was added to the appropriate wells. 3ul of copy control dilution series was added to specific wells as a control. The reactions were run in an ABI7900 (Applied Biosystems) using the following cycling conditions:

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Step	Temperature	Time
1	50°C	2 min
2	95°C	10 min
3	95°C	15 sec
10 4	60°C	1 min
5	Goto step 3, repeat 40 times	

Data was analysed using SDS2.0 software (Applied Biosystems).

15 **Example 3: COT102 Detection via PCR**

3.1 Genomic DNA extraction

Genomic DNA from COT102 was extracted as described in Example 2.1.

3.2 Multiplex PCR Zygoty Test

20 PCR primers were designed to bind to cotton genomic DNA sequence upstream of the site at which the COT102 cassette inserted (SEQ ID NO: 18); the COT102 cassette sequence itself (SEQ ID NO: 19); and cotton genomic DNA sequence which is replaced when the COT102 sequence is inserted (SEQ ID NO: 20). When the COT102 insert is present, primer pairs SEQ ID NO: 18 and 19 amplify a PCR fragment of 962bp in size. A 50ul
 25 PCR reaction was set up for each sample to be tested as follows:

	1x JumpState ReadyMix REDTaq PCR (Sigma P-1107)	25ul
	40 pmole primer 1 (SEQ ID NO: 18)	4ul
	40 pmole primer 2 (SEQ ID NO: 19)	4ul
30	40 pmole primer 3 (SEQ ID NO: 20)	4 ul
	40 ng genomic DNA	4ul
	ddH2O	9ul

The PCR reactions were heated in a thermocycler at 94°C for 2 minutes, followed by 35 cycles as follows: 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute. The reaction was completed by heating at 72°C for 5 minutes.

5 3.3 *Analysis*

PCR reactions were run on an agarose gel, and DNA bands visualised under UV light after staining with ethidium bromide. The presence of 3 bands indicated that the sample was a COT102 homozygote plant; 2 bands (one of which being 962bp in size) indicated that the sample was a COT102 heterozygote plant; 2 bands (with no band of 962bp in size) indicated that the sample was a homozygote wild type cotton plant.

3.4 *Event specific PCR*

One PCR primer was designed to bind towards the 3' end of the VIP3A gene (SEQ ID NO: 3). Another PCR primer was designed to bind to the complementary strand of the flanking genomic DNA sequence downstream of the 3' end of the COT102 insertion site (SEQ ID NO: 4). These primers were used together in a PCR reaction using COT102 genomic DNA resulting in the amplification of an 800bp fragment. When the primers were used in a PCR reaction using a Coker312 non-transformed cotton genomic DNA sample, no fragment was amplified.

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In a second pair of primers, one primer was designed to bind to the hygromycin gene (SEQ ID NO: 19) and the other primer was designed to bind to the flanking genomic DNA sequence upstream of the 5' end of the COT102 insertion site (SEQ ID NO: 18). These primers were used together in a PCR reaction using COT102 genomic DNA resulting in the amplification of a 962bp fragment. When the primers were used in a PCR reaction using a Coker312 non-transformed cotton genomic DNA sample, no fragment was amplified.

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Example 4: COT102 Detection via Southern Blot

30 4.1 *DNA extraction for use in Southern Blotting*

Approximately 5 to 10 grams of plant tissue was ground in liquid nitrogen using a mortar and pestle. The plant tissue was resuspended in 12.5ml extraction buffer A (0.2M Tris pH8.0, 50mM EDTA, 0.25M NaCl, 0.1% v/v B-mercaptoethanol, 2.5% w/v Polyvinyl-

pyrrolidone), and centrifuged for 10 minutes at 4,000 rpm (2755g). After discarding the supernatant the pellet was resuspended in 2.5ml Extraction Buffer B (0.2M Tris pH8.0, 50mM EDTA, 0.5M NaCl, 1% v/v B-mercaptoethanol, 2.5% w/v Polyvinyl-pyrrolidone, 3% Sarkosyl, 20% Ethanol) and incubated at 37°C for 30 minutes. During incubation, the sample was mixed once with a sterile loop. After incubation an equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed gently by inversion, and centrifuged for 20 minutes at 4,000 rpm. The aqueous layer was collected, and 0.54 volume isopropanol added followed by centrifugation for 5 minutes at 4,000 rpm to precipitate the DNA. The supernatant was discarded and the DNA pellet resuspended in 500ul TE. In order to degrade any RNA present, the DNA was incubated at 37°C for 30 minutes with 1ul 30mg/ml RNase A, centrifuged for 5 minutes at 4,000 rpm and precipitated by centrifugation at 14,000 rpm for 10 minutes in the presence of 0.5 volume 7.5M ammonium acetate and 0.54 volume isopropanol. After discarding the supernatant, the pellet was washed with 500ul 70% ethanol and allowed to dry before resuspending in 100ul TE.

4.2 Restriction enzyme digests

The DNA was quantified using a spectrophotometer or fluorometer (using 1xTNE and Hoechst dye). Suitable enzyme digests were prepared using 8ug DNA per digest in a total volume of 50ul. Digests included *BamHI*, *EcoRI*, *EcoRV*, *HindIII*, *NcoI*, *SacI*, *ScaI*, *SpeI* and *PstI*, both alone and in combination. In particular, a *BamHI* and *EcoRI* double digest was used to detect the intactness of the VIP3A gene; a *BamHI* and *EcoRV* double digest was used to detect VIP3A locus number and intactness of the hygromycin gene; and a *BamHI* single digest was used to detect VIP3A locus number. Digests were incubated overnight at the appropriate temperature for each enzyme. Samples were spun in a speed vacuum to reduce the volume to 30ul.

4.3 Gel electrophoresis

Bromophenol blue loading dye was added to each sample from 4.2 above, and each sample loaded on a 0.8% TBE agarose gel containing ethidium bromide. The gel was run at 60 volts overnight.

The gel was washed in 0.25M HCl for 15 minutes to depurinate the DNA, and then washed with water. A Southern Blot was set up as follows: 20 sheets of thick dry blotting paper was placed in a tray and 4 sheets of thin dry blotting paper placed on top. One sheet of thin blotting paper was pre-wet in 0.4M NaOH and placed on top of the stack, followed by
5 a sheet of Hybond-N+ transfer membrane (Amersham Pharmacia Biotech, #RPN303B), also pre-wet in 0.4M NaOH. The gel was placed on top ensuring that there were no air bubbles between the gel and the membrane. Three further sheets of pre-soaked blotting paper were placed on top of the gel and the buffer tray filled with 0.4M NaOH. Connecting the gel stack with the buffer tray using a wick pre-soaked in 0.4M NaOH
10 started the transfer of DNA to the membrane. DNA transfer took place for approximately 4 hours at room temperature. Following transfer, the Hybond membrane was rinsed in 2x SSC for 10 seconds and the DNA bound to the membrane via UV cross-linking.

4.4 Hybridisation

15 A suitable DNA probe was prepared by PCR. 25ng probe DNA in 45ul TE was boiled for 5 minutes, placed on ice for 7 minutes then transferred to a Rediprime II (Amersham Pharmacia Biotech, #RPN1633) tube. After addition of 5ul P32-labelled dCTP to the Rediprime tube, the probe was incubated at 37°C for 15 minutes. The probe was purified by centrifugation through a microspin G-50 column (Amersham Pharmacia Biotech, #27-
20 5330-01) according to the manufacturers instructions to remove unincorporated dNTPs. The activity of the probe was measured using a scintillation counter.

The Hybond membrane was pre-hybridised by wetting with 20ml pre-warmed Church pre-hybridisation solution (500mM NaPO₄, 1mM EDTA, 7% SDS, 1% BSA) at 65°C for 30
25 minutes. The labelled probe was boiled for 5 minutes, and placed on ice for 10 minutes. An appropriate amount of probe (1 million counts per 1ml pre-hybridisation buffer) was added to the pre-hybridisation buffer and hybridisation occurred at 65°C overnight. The following day, the hybridisation buffer was discarded, and following a rinse with 20ml Church Wash Solution 1 (40mM NaPO₄, 1mM EDTA, 5% SDS, 0.5% BSA), the
30 membrane washed in 150ml Church Wash Solution 1 at 65°C for 20 minutes. This process was repeated twice with Church Wash Solution 2 (40mM NaPO₄, 1mM EDTA, 1% SDS). The membrane was exposed to a phosphor screen or X-ray film to detect where the probe has bound.

Example 5: COT102 Detection via ELISA

5.1 Protein extraction

Cotton tissue for analysis was harvested and frozen at -70°C. Fresh tissue was ground to a fine powder and weighed into a labelled polypropylene tube. Extraction buffer (100mM Tris, 100mM Sodium Borate, 5mM MgCl, 0.05% Tween 20, 0.2% Sodium Ascorbate, Water, pH 7.8, 1mM AEBSF, 0.001mM Leupeptin) was added to the sample in a ratio of 2:1 (volume extraction buffer : sample fresh weight) for fresh tissue or 30:1 (volume extraction buffer : sample dry weight) for lyophilised tissue. The sample was vortexed and homogenised using a Brinkman PT 10/35 Polytron equipped with a PTA 10TS foam-reducing generator, until the mixture became liquefied. Extracts were centrifuged at 10,000 x g for 15 minutes. The protein extract supernatant was stored at 2-8°C.

5.2 ELISA protocol

The ELISA procedure used standard techniques as follows. A 96-well plate was soaked in ethanol for 2 hours, and air-dried. The plate was coated with 50ul goat anti-VIP3A antibody per well and incubated overnight at 2-8°C. After washing three times with 1X ELISA wash solution (100mM Tris, 0.5% Tween-20, 75mM NaCl, pH8.5), the plate was dried briefly by tapping upside down on a paper towel. 150ul blocking solution (10mM NaPO₄, 140mM NaCl, 1% BSA, 0.02% Sodium Azide, titrated to pH7.4 with monobasic NaPi and dibasic NaPi) was added to each well followed by incubation at room temperature for 45 minutes. The plate was washed 3 times as described above.

VIP3A standards and protein extract samples were applied to appropriate wells of the plate in triplicate, 50ul total volume per well. The plate was incubated at 2-8°C for 1 hour 30 minutes, followed by room temperature for a further 30 minutes. The plate was washed three times with ELISA wash solution, and then incubated at 35-39°C for 1 hour with 50ul rabbit anti-VIP3A antibody per well. The plate was washed three times with ELISA wash solution, and incubated at room temperature for 30 minutes with 50ul donkey anti-rabbit alkaline phosphatase per well. Following a further three washes with ELISA wash solution, 50ul phosphatase substrate solution was added per well and the plate incubated for 30 minutes at room temperature. The reaction was stopped by addition of 50ul 3M NaOH per well. The absorbance of the solution in each well was measured at 405nm using

a Ceres 900C multiwell plate reader and the results analysed using KC3 Curve fitting software (Bio-Tek Instruments Inc.). The concentration of VIP3A in the samples was calculated by reference to the VIP3A protein standards.

5 **Example 6: COT102 detection via DipStick**

6.1 *Protein extraction*

A piece of leaf tissue approximately 2 cm² was placed in a tube containing extraction buffer. A plastic stirrer was used to extract protein from the tissue, by cutting into and mascerating the tissue.

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6.2 *Dipstick test*

A test strip was placed into the tube and incubated for 5 - 10 minutes for the result to develop. The test strip comprised a first band at which anti-VIP3A antibody was bound, and a second band at which a control antibody was bound. After incubation, a double red
15 line in the result window of the test strip indicated that VIP3A was present. The lower line indicated the presence of Vip3A protein while the upper line was a control indicating that the assay was working correctly.

Example 7: COT102 Detection via Insect Bioassay

20 7.1 *Leaf biosassays*

Leaf assays were performed on Fall Army Worm (*Spodoptera frugiperda*), Cotton Boll Worm (*Helicoverpa zea*) and Tobacco Budworm (*Heliothis virescens*) as follows:

Pads were soaked with 300ul to 500ul distilled water and placed into Gelman dishes. Leaf pieces measuring between approximately 0.5 square inches and 0.75 square inches were
25 excised from cotton plants 8 to 12 inches in height, and placed on the pads. Between 8 and 10 insect larvae were placed in each dish and a lid fitted. The dishes were incubated at 28°C. On the third and sixth days after infestation, damage to the leaf in each dish was scored and compared with the control plants.

30 7.2 *Boll bioassays*

Four absorbent pads were saturated with water and placed inside a large plastic cup. Three extra thick glass filters, each soaked with 100ul distilled water, were placed in a smaller plastic cup, which was then seated inside the larger cup. A 1.25 inch long boll was

excised, immersed in 10mg/ml to 20mg/ml Nystatin and placed on the filters in the small cup. 50 insect larvae were placed on the square or boll and a lid attached to the larger cup. The squares or bolls were re-infested with 50 more larvae after 7 days.

- 5 The experiment was incubated at room temperature for approximately 3 weeks. The bolls were then cut open to determine damage. Damage to the boll was compared to the control samples.

7.3 Lyophilised leaf bioassays

- 10 Bioassays using freeze-dried leaf tissue were performed on *Heliothis virescens* as follows:

Terminal leaves were snap-frozen on dry-ice at time of picking and lyophilised overnight. The freeze dried tissue was ground in a mortar and pestle to a fine powder and resuspended
15 in 0.2% agar solution to make an 8% (0.08g/ml) suspension of leaf powder. The suspension was overlaid on top of artificial insect diet in 96-well plates and left to dry. A single neonate insect larva was introduced into each well and the plates sealed. The plates were incubated at 28°C. On the sixth day after infestation, larval mortality was scored and compared with control samples. Results obtained were as follows:

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variety	% leaf powder suspension	% larval mortality (mean of 5 tests)
Coker 312	8	6.7
COT102	8	98.3